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Pathogenesis of 1918 Pandemic and H5N1 Influenza Virus Infections in a Guinea Pig Model: Antiviral Potential of Exogenous Alpha Interferon To Reduce Virus Shedding▽

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Although highly pathogenic avian influenza H5N1 viruses have yet to acquire the ability to transmit efficiently among humans, the increasing genetic diversity among these viruses and continued outbreaks in avian species underscore the need for more effective measures for the control and prevention of human H5N1 virus infection. Additional small animal models with which therapeutic approaches against virulent influenza viruses can be evaluated are needed. In this study, we used the guinea pig model to evaluate the relative virulence of selected avian and human influenza A viruses. We demonstrate that guinea pigs can be infected with avian and human influenza viruses, resulting in high titers of virus shedding in nasal washes for up to 5 days postinoculation (p.i.) and in lung tissue of inoculated animals. However, other physiologic indicators typically associated with virulent influenza virus strains were absent in this species. We evaluated the ability of intranasal treatment with human alpha interferon (α-IFN) to reduce lung and nasal wash titers in guinea pigs challenged with the reconstructed 1918 pandemic H1N1 virus or a contemporary H5N1 virus. IFN treatment initiated 1 day prior to challenge significantly reduced or prevented infection of guinea pigs by both viruses, as measured by virus titer determination and seroconversion. The expression of the antiviral Mx protein in lung tissue correlated with the reduction of virus titers. We propose that the guinea pig may serve as a useful small animal model for testing the efficacy of antiviral compounds and that α-IFN treatment may be a useful antiviral strategy against highly virulent strains with pandemic potential.

Since 2003, influenza A viruses of the H5N1 subtype have caused devastating outbreaks in poultry in Asia, Africa, and Europe, resulting in over 400 human infections, with an overall case fatality rate of 60% (1). The increasing persistence and genetic diversity of H5N1 viruses in poultry with concomitant human infection indicate that H5N1 viruses remain a pandemic threat (3). Despite evidence for limited human-to-human transmission, these viruses have yet to exhibit sustained transmission among humans (30, 50, 77). If H5N1 viruses were to acquire this ability, the resulting pandemic could be unusually severe, requiring multiple control measures to limit the morbidity and mortality associated with a pandemic virus. Vaccination remains the primary method of reducing the morbidity associated with seasonal influenza virus infection. Due to the diversity in circulating H5N1 viruses and the overall timeline for manufacturing, antigenically well-matched vaccines may not be available in the initial stages of an H5N1 pandemic (67, 69).

Currently, FDA-approved influenza virus antivirals consist of the adamantane compounds (amantidine/rimantidine) and the neuraminidase inhibitors oseltamivir and zanamivir (20, 72). However, widespread adamantine resistance was recently documented among seasonal H1N1 and H3N2 strains, in addition to a majority of clade 1 and some clade 2 H5N1 isolates from Southeast Asia (2, 9, 10, 12, 81). Oseltamivir-resistant H5N1 and H1N1 isolates have also been reported (11, 35, 62). Given the potential for resistance to existing antivirals, the identification of additional therapeutics that may limit the replication of H5N1 viruses and thereby reduce morbidity and transmission in the early stages of a pandemic is a high priority.

The interferon (IFN) response is a critical component of the host innate antiviral response, and compounds that trigger or enhance this response are already in use clinically to treat a number of viral infections (16). Accumulated research suggests that engagement of the IFN response prior to infection may be a viable therapeutic strategy to control influenza virus infection. In the context of a natural infection, influenza A viruses induce IFN-α/β in mice and humans, but the level of induction is highly variable and strain dependent (4, 16, 22, 23, 74, 84). Furthermore, mice rendered IFN deficient through deletion of the IFN-α/β receptor or STAT1 show increased viral titers and extrarespiratory spread following infection with H1N1 viruses and display increased morbidity and mortality following H5N1 virus infection (17; K. Szretter et al., submitted for publication). These studies clearly establish the IFN pathway as critical for the control of influenza virus infection in mice.

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The Mx GTPase is one of many antiviral proteins induced during the IFN response (57). Mx has been shown to significantly contribute to the control and respiratory restriction of highly pathogenic influenza virus strains and is necessary for the establishment of an influenza-resistant state following prophylactic treatment with exogenous IFN (59, 66, 76). However, standard laboratory mouse strains, including BALB/c and C57BL/6, carry deletions in this gene (65). In comparison, the guinea pig, recently found to be an effective model for the study of influenza virus infection and transmission, possesses a functional Mx gene (25), highlighting this model as ideal for studying the IFN response to influenza virus infection (39).

Various species have been used as animal models for influenza virus infection (reviewed in reference 78). However, the most common mammalian model used for influenza virus research, the mouse, is not susceptible to infection with unadapted human influenza A viruses and does not shed virus from the respiratory tract. Therefore, there is a need to develop additional permissive small animal models of influenza virus infection that exhibit virus shedding. Serial samples collected from such animal models allow the investigator to determine both the titer and duration of virus shedding from individual animals at multiple times without euthanasia. While the guinea pig model has been shown to support the replication of contemporary human influenza virus strains, their susceptibility to highly pathogenic influenza virus strains is currently unknown (40). In this study, we assessed the virulence of the highly pathogenic 1918 pandemic and H5N1 viruses in addition to low-pathogenicity avian and human H1N1 viruses in the guinea pig model. We demonstrate that guinea pigs are naturally susceptible to infection with avian and human influenza viruses. Highly pathogenic viruses, in comparison with viruses of low pathogenicity, were isolated from the upper respiratory tracts of guinea pigs at later times postinfection and induced more severe lung lesions. The ability of human IFN to inhibit virus replication and to limit virus-induced pathology following infection with both 1918 and H5N1 viruses was examined. We found that prophylactic IFN treatment was sufficient to reduce or prevent the replication of highly pathogenic H5N1 and H1N1 viruses in the upper airway.

MATERIALS AND METHODS

Viruses. Two influenza A viruses of high pathogenicity, the clade 1 avian H5N1 virus A/Thailand/16/04 (H5N1) and the reconstructed 1918 H1N1 pandemic virus (1918) (73), and two influenza A (H1N1) viruses of low pathogenicity, the avian virus A/Dk/Alb/35/76 (Dk/Alb) and the human isolate A/Tx/36/91 (Tx91), were used in this study. Avian viruses were propagated in the allantoic cavity of 10-day-old embryonated hen's eggs at 37°C for 24 h to 30 h. Human influenza virus strains were incubated for an additional 10 h to 18 h. Allantoic fluid was pooled from multiple eggs, clarified by centrifugation, and frozen at −70°C until use. The 50% egg infectious dose (EID_{50}) was determined by serial titration of virus stock in eggs, and EID_{50}/ml values were calculated according to the method of Reed and Muench (55). Human virus stocks were grown in MDCK cells as described previously (73), with viral titers determined by standard plaque assay. All experiments using H5N1 or 1918 virus were conducted under biosafety level 3 containment (http://www.cdc.gov/OD/ohs/biosfty/bmbl5/bmbl5toc.htm), including enhancements required by the U.S. Department of Agriculture and Select Agent Program (56).

Animals. Female Hartley strain guinea pigs weighing 300 to 350 g were obtained from Charles River Laboratories (Frederick, MD). During challenge studies, animals were housed in a Duo-Flo Bioclean mobile clean room (Lab Products, Seaford, DE). Animals were allowed free access to food and water and were maintained in 12-h light-dark cycles. Prior to each manipulation, guinea pigs were anesthetized by intramuscular injection of a mixture of ketamine (30 mg/kg of body weight) and xylazine (2 mg/kg). Animal research was conducted under the guidance of the Centers for Disease Control and Prevention's Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility.

Pathotyping studies. For assessment of virus pathogenicity, three or four guinea pigs were inoculated intranasally with 10^6 EID_{50} of virus by instillation of 150 μl of diluted virus into each nostril. Implantable subcutaneous temperature transponders were applied as previously described (44), and guinea pigs were monitored daily for changes in weight and temperature to assess virus-induced morbidity. Nasal washes were collected on days 1, 3, 5, and 7 p.i. to assess replication kinetics in the upper airway for each virus. Nasal washing was performed by instillation of 1 ml of phosphate-buffered saline (PBS) containing 100 μg/ml penicillin-streptomycin (Gibco, Grand Island, NY), 100 μg/ml gentamicin (Gibco), and 1% bovine serum albumin (Gibco) into the nostrils and collection of liquid runoff into a sterile petri dish. Nasal washes were immediately frozen on dry ice and stored at −80°C until titer determination.

To determine the ability of each virus to replicate in the lower respiratory tract or outside the respiratory tract, lung and spleen tissues were harvested from three additional animals on day 3 p.i. Prior to collection of lung and spleen samples, animals were deeply anesthetized as described above, exsanguinated, and humanely euthanized by intracardiac injection with Beuthanasia-D solution (Schering-Plough Animal Health Corporation, Kenilworth, NJ) (1 ml/kg). Tissue samples for titration were removed and immediately frozen on dry ice. For titer analysis, tissue samples were homogenized in 1 ml of PBS containing antibiotics (Gibco). Tissue homogenates were clarified by centrifugation, and virus titer was determined by standard plaque assay or by serial dilution in embryonated chicken eggs as described previously (44). At the time of harvest, the remaining tissues were fixed in 10% formalin for evaluation of histopathology. From each guinea pig, one piece of spleen and one to four pieces of lung tissue were immersed in 10% neutral buffered formalin solution, routinely processed, and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin, and duplicate 5-μm sections were immunohistochemically stained to demonstrate influenza A virus nucleoprotein, using a mouse monoclonal antibody as previously described (53, 70).

IFN treatment studies. The IFN used in this study is a recombinant human αB/D protein that has previously been described and shown to be highly active in mice (14, 24). The recombinant protein was stored lyophilized at 4°C and reconstituted immediately prior to use. A dose of IFN corresponding to 500,000 U/kg of the average weight of all animals in each experiment, previously shown to effectively induce an antiviral state among treated animals (76), was given intranasally in a volume of 300 μl, with 150 μl instilled into each nostril. IFN was diluted in PBS containing antibiotics (Gibco). In experiments involving virus challenge, the challenge dose varied between 10^3 and 10^6 PFU or EID_{50}, as indicated. For single-treatment studies, IFN was provided 12 h prior to virus inoculation. For multiple-treatment studies, guinea pigs were treated with 500,000 U/kg/day 1 day before inoculation, 12 h prior to inoculation (24 h after the initial IFN treatment), and every other day for 7 days p.i. Seroconversion of untreated and treated guinea pigs was determined by the testing of sera collected at 21 days p.i. by hemagglutination inhibition (HI) assay with 1% horse (H5N1 and Dk/Alb viruses) or 1% turkey (1918 and Tx91 viruses) red blood cells as described previously (68).

Mx protein expression analysis. Mx protein expression in guinea pig lung tissues was examined following a single IFN treatment of 500,000 U/kg, H5N1 virus inoculation, or a combination of IFN treatment followed by H5N1 virus challenge. In the last group, animals were treated with IFN or PBS 12 h prior to inoculation with 10^3 EID_{50} of H5N1 virus, as described above. Individual guinea pigs were euthanized on days 1, 2, and 3 posttreatment/postinfection, and collection of lung tissues was performed as described above. Individual 10-mm lung pieces were collected from each animal from areas representing four quadrants (upper and lower right and left sides). Western blot analysis to detect Mx protein expression levels in extracted lung tissue was carried out using previously described methods (84). Briefly, at each indicated time point, lung tissue samples were homogenized in 500 μl PBS containing a protease inhibitor cocktail (Sigma, St. Louis, MO). Following centrifugation, pellets were resuspended in 500 μl Laemmli buffer (Bio-Rad, Hercules, CA) and boiled for 10 min. Protein content was assayed spectrophotometrically with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE), and equal amount of proteins were loaded in a sodium dodecyl sulfate-polyacrylamide gel. Immunoreactive proteins were detected using antibodies to β-actin (Sigma) or MxA antibody and incubated for 45 min at room temperature or overnight at 4°C, respectively. The MxA antibody used was a mouse monoclonal antibody, M143, against human MxA that was

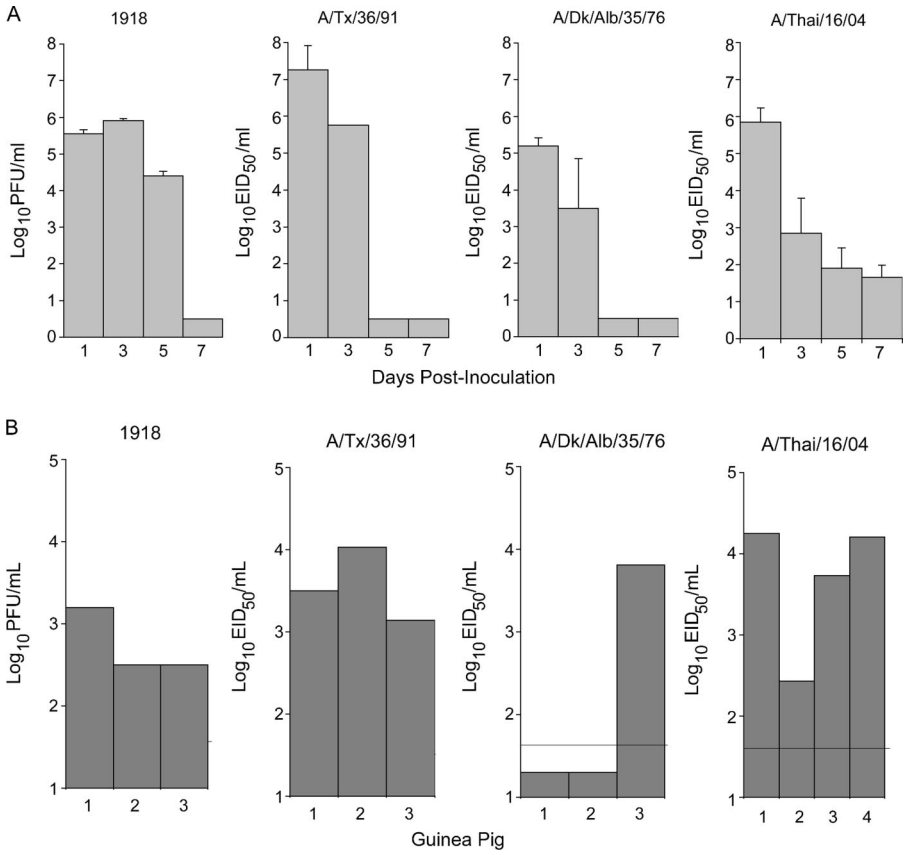

FIG. 1. Replication of H1N1 and H5N1 viruses in the upper and lower respiratory tracts of guinea pigs. Following intranasal inoculation of guinea pigs, virus replication was assessed by titration of nasal washes (A) and lung tissue samples (B). Nasal washes were collected on days 1, 3, 5, and 7 p.i. with 10^6 EID_{50} or 10^6 PFU of virus. (A) Mean titers for three animals are plotted, and error bars represent standard deviations from the means. (B) Three additional animals were inoculated, and lung tissue samples were collected for titration on day 3 p.i. Virus titers determined from the homogenized lungs were plotted.

generated from a hybridoma culture supernatant (kindly provided by Otto Haller and Georg Kochs, University of Freiburg, Freiburg, Germany). Primary antibody binding was detected using a horseradish peroxidase-conjugated anti-mouse immunoglobulin G heavy-plus-light-chain polyclonal antibody (Cell Signaling Technologies, Boston, MA) according to the manufacturer's protocol.

RESULTS

Infection of guinea pigs with highly virulent human and avian influenza viruses. To investigate the pathogenicity of highly virulent avian and human influenza viruses in the guinea pig model, female Hartley strain guinea pigs were inoculated with four viruses that differ in their pathogenicity in mice and ferrets (44, 75). All viruses tested productively infected the guinea pigs, with peak virus titers in nasal wash of $>10^5$ PFU/ml or EID_{50}/ml on day 1 p.i. (Fig. 1; Table 1). Tx91 and Dk/Alb viruses persisted in nasal washes through day 3 p.i. but were below detectable levels by day 5 p.i. In contrast, viral titers of the highly virulent 1918 and H5N1 viruses persisted in nasal washes through days 5 and 7 p.i., respectively (Fig. 1). To evaluate the replication of these viruses in the lower respiratory tract of guinea pigs, lung tissue was collected from three or four guinea pigs per virus on day 3 p.i., when influenza virus replication is at a peak in other mammalian models (44). All four influenza viruses tested replicated in guinea pig lungs without prior host adaptation, with mean lung viral titers that ranged from $10^{2.9}$ to $10^{3.8}$ EID_{50}/g (Table 1). In addition to evaluation of virus titers, physiologic indicators of viral infection were monitored for the duration of virus shedding. In previous studies, both 1918 and H5N1 viruses have been shown to be highly pathogenic in mice and ferrets, causing high morbidity and mortality (44, 73). In contrast, infection of guinea pigs with the highly virulent influenza viruses did not cause morbidity (Table 1). Most strikingly, infection of guinea pigs with 1918 virus did not result in any weight loss at any time

TABLE 1. Pathogenicity of H1N1 and H5N1 viruses in guinea pigs

Virus	Subtype	Weight loss (%)[a]	Mean titer	
			Nasal wash (log_{10} EID_{50}/ml or PFU/ml)	Lung (log_{10} EID_{50}/g or PFU/g) (no. of animals with titer/ total no. of animals)
1918	H1N1	0.0	5.6	2.9 (3/3)
A/Thailand/16/04	H5N1	7.3	6.0	3.3 (3/4)
A/Duck/Alberta/ 35/76	H1N1	3.2	5.2	3.8 (1/3)
A/Tx/36/91	H1N1	1.9	7.4	3.4 (3/3)

[a] Maximum mean weight change observed during the first 9 days p.i.

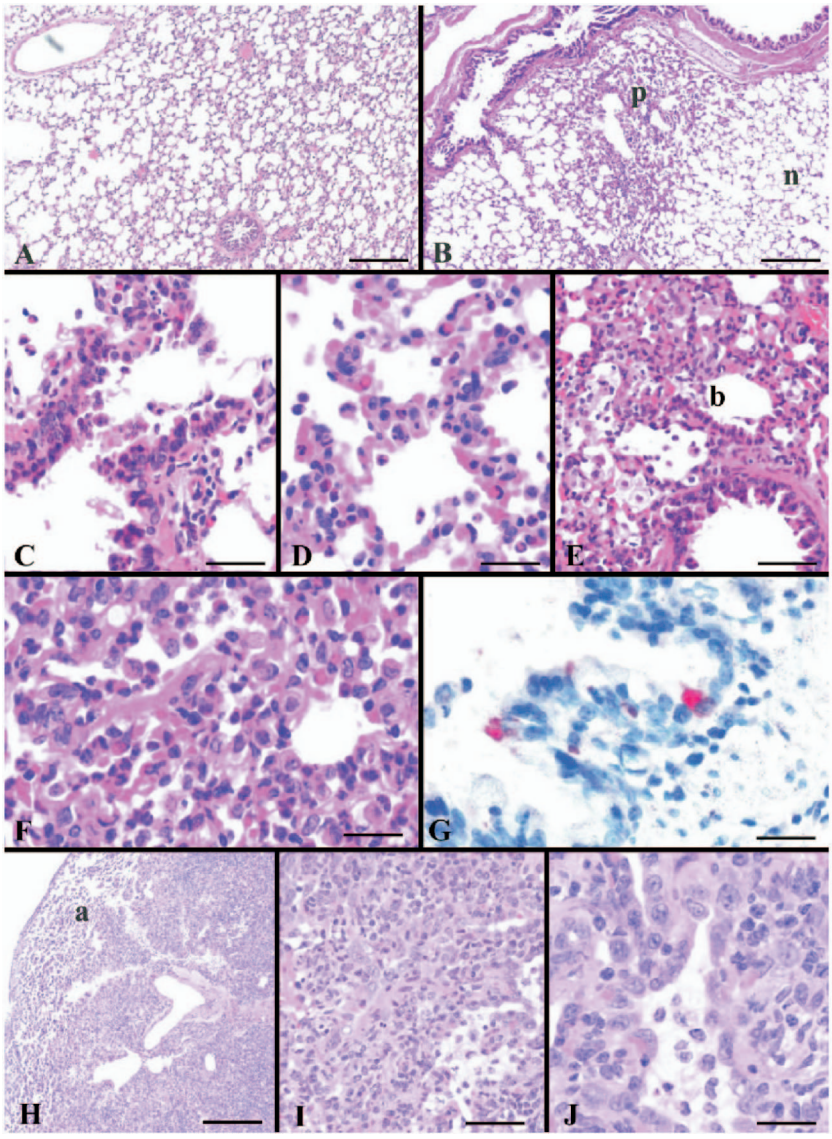

FIG. 2. Photomicrographs of hematoxylin- and eosin-stained and immunohistochemically stained tissue sections from guinea pigs. (A) Healthy, sham-inoculated lung at 3 days p.i. Bar = 250 μm. (B) Mild interstitial pneumonia (p) adjacent to a large bronchiole in the proximal lung lobe area and healthy lung tissue (n) in the distal lung lobe area in a Tx/91 H1N1 virus-infected animal at 3 days p.i. Bar = 250 μm. (C) Mild bronchiolitis with epithelial necrosis and luminal heterophils in a Tx/91 H1N1 virus-infected animal at 3 days p.i. Bar = 50 μm. (D) Thickening of alveolar walls from histiocytes and heterophils in a Dk/Alb H1N1 virus-infected animal at 3 days p.i. Bar = 25 μm. (E) Occlusion of bronchiole (b) and adjacent alveoli filled with foamy macrophages in a Dk/Alb H1N1 virus-infected animal at 3 days p.i. Bar = 50 μm. (F) Severe bronchointerstitial pneumonia with hyaline membranes in alveoli in a Dk/Alb H1N1 virus-infected animal at 3 days p.i. Bar = 20 μm. (G) Influenza viral antigen in epithelium of a large bronchiole in a Tx/91 H1N1 virus-infected animal at 3 days p.i. Bar = 20 μm. (H) Severe interstitial pneumonia with atelectasis (a) in the peripheral lung lobe area in a 1918 H1N1 virus-infected animal at 3 days p.i. Bar = 300 μm. (I) Lymphohistiocytic interstitial pneumonia with early fibroplasia in a 1918 H1N1 virus-infected animal at 3 days p.i. Bar = 55 μm. (J) Bronchiolar collapse with hyperplastic regenerative epithelium and adjacent severe interstitial pneumonia in a 1918 H1N1 virus-infected animal at 3 days p.i. Bar = 30 μm.

point following infection. H5N1 virus infection resulted in modest weight loss (7.3%), although 100% of animals infected with this virus recovered to reach their initial body weight by day 13 p.i. (data not shown). In addition, no increase in lethargy, as determined by calculation of a relative inactivity index (86), was observed for either virus. Collectively, these observations suggest that while they are able to replicate efficiently in the upper and lower respiratory tracts of guinea pigs, 1918 and H5N1 influenza viruses are not highly pathogenic in this model.

Guinea pig lung pathology. Many influenza A viruses of high pathogenicity induce severe histopathological changes in pulmonary and extrapulmonary tissues of mouse and ferret models (44, 71). In the current study, lesions were lacking in healthy, sham-inoculated guinea pigs (Fig. 2A). Both high- and low-pathogenicity influenza virus strains induced lesions only

in the lungs, producing minimal to moderate bronchointerstitial pneumonia, with the greatest severity adjacent to the hilus in the dorsal region of each lung lobe and with the periphery of the lung lobe having the least severe pneumonia or atelectatic normal lung parenchyma (Fig. 2B and H). Necrosis of bronchiolar epithelium with associated histiocytic-to-heterophilic inflammation and occasional luminal casts were present in terminal bronchioles (Fig. 2C), but such lesions were less common in bronchi, and many bronchioles and bronchi were normal. Alveolar walls were thickened with infiltrates of histiocytes and, less commonly, heterophils, with some edema, and rarely with fibrin (Fig. 2D). Some alveolar ducts were occluded with cellular debris, and the adjacent alveoli were filled with large foamy macrophages (Fig. 2E), with occasional mixed inflammatory cells obscuring the alveolar walls or, most rarely, hyaline membranes present (Fig. 2F), which indicates severe damage to the alveolar type 1 pneumocytes. Influenza viral antigen was demonstrated infrequently, mainly in bronchiolar epithelial cells or histiocytes within areas of pneumonia (Fig. 2G).

The most severe pneumonia was in guinea pigs inoculated with 1918 H1N1 virus (Fig. 2H, I, and J), followed by H5N1 virus and Dk/Alb H1N1 virus, with the least severe lesions occurring with Tx/91 H1N1 infection. Taken together, these findings show that influenza viruses of both high and low pathogenicity can induce pulmonary lesions in guinea pigs but that highly pathogenic viruses induce the most severe lesions. In addition, guinea pigs had comparatively fewer severe lesions than those reported for ferrets or mice inoculated intranasally with the same viruses (44, 75), and the guinea pig airway epithelium of bronchi and upper bronchioles was only mildly susceptible to virus infection and necrosis compared to the more frequent and severe necrosis seen in the terminal bronchioles.

Induction of MxA protein following treatment of guinea pigs with intranasal IFN. The controlled virus replication of virulent influenza virus strains and the lack of severe lung lesions in guinea pigs suggest that this animal model is suited for study of the IFN response to influenza virus infection. It is possible that the functional Mx proteins of guinea pigs may limit influenza virus virulence and that this model could be used for the development of antiviral treatments against influenza viruses with pandemic potential. To determine whether exogenous IFN was capable of inducing an antiviral state in this species, we examined the induction of the Mx protein following a single treatment of recombinant human IFN-α. Western blots of extracted lung tissue from IFN-treated and/or H5N1-infected guinea pigs were performed with the M143 monoclonal antibody directed against the MxA protein (Fig. 3). As early as 24 h following intranasal treatment with 500,000 U/kg of recombinant IFN, a dose of IFN previously shown to induce a potent antiviral state in Mx-positive laboratory mice (76), a robust induction of Mx protein was observed in IFN-treated animals compared with PBS-treated animals (Fig. 3, top panels). High levels of Mx protein were also observed at 48 h posttreatment, at which point protein levels slightly declined; reduced expression was observed at 72 h posttreatment. A contrasting Mx profile was observed for guinea pigs receiving an H5N1 virus infection. We consistently found that intranasal infection with H5N1 virus resulted in an induction of Mx protein that was not observed until 48 h p.i., with negligible or no Mx protein expression at the 24-h time period (Fig. 3, bottom panels). Mx protein expression remained high through day 3 post-H5N1 virus infection. In animals that received a single dose of exogenous IFN followed by H5N1 virus challenge, Mx protein was induced at 24 h p.i. and observed through 72 h p.i. Interestingly, in comparison to the experimental groups that received IFN only or H5N1 virus challenge only, this group consistently displayed a reduction in Mx protein expression at day 3 p.i. Taken together, these results show that both IFN treatment and H5N1 virus challenge can induce potent antiviral Mx protein expression in guinea pig lung tissue.

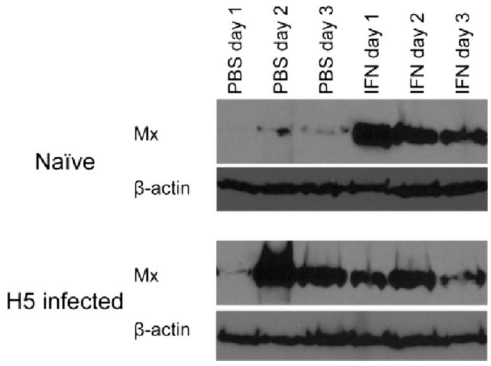

FIG. 3. Analysis of Mx protein expression in IFN- and PBS-treated animals. Mx protein expression in the guinea pig lung was examined by Western blotting following a single treatment with either PBS or 500,000 U/kg of exogenous IFN. Mx protein samples were analyzed on days 1, 2, and 3 post-IFN treatment (top). Mx expression was also examined in H5N1 virus-infected lung tissue (bottom). In this case, animals were treated with IFN or PBS 12 hours prior to inoculation with 10^3 EID_{50} of H5N1 virus. Samples were taken beginning at 24 hours postinfection (day 1) and continuing until day 3 p.i. Beta-actin was used as a loading control.

Reduction of H5N1 and 1918 virus titers following multiple treatments of guinea pigs with recombinant human IFN. Having demonstrated that exogenous IFN treatment could induce an antiviral response in guinea pigs, we next examined the ability of IFN treatment to reduce viral titers of highly pathogenic avian and human influenza virus strains in this species. Animals were treated intranasally with recombinant human IFN-α prior to and following virus inoculation on days −1, 0, 1, 3, 5, and 7 p.i. On day 0, approximately 12 h following IFN treatment, four animals were inoculated intranasally with 10^3 PFU or EID_{50} of 1918 or H5N1 virus, respectively. This reduced dose of virus was chosen based on previous studies using seasonal influenza virus strains in guinea pigs wherein a guinea pig infectious dose was determined (40). As expected, this dose of virus was sufficient to infect untreated control animals, as virus was recovered from nasal washes from all untreated animals, and virus-specific HI antibody titers were present in sera collected from all animals at 21 days p.i. (Table 2). Strikingly, multiday IFN treatment blocked virus infection in 50% of animals inoculated with this low dose (10^3) of either 1918 or H5N1 virus (Fig. 4A, B, E, and F; Table 2). Two of four IFN-treated animals in each group possessed detectable anti-HI serum antibodies that largely correlated with detection of infectious virus recovered from nasal washes (2/4 animals inoculated with 1918 virus and 1/4 animals inoculated with H5N1

TABLE 2. Reductions in viral titers observed following IFN treatment of guinea pigs

Virus	Dose	Treatment[a]	Mean peak titer in nasal wash[b]	No. of animals with seroconversion/ total no. of animals (HI titer)
1918	10^6 PFU	None	5.3 (1)	4/4 (160–320)
	10^6 PFU	IFN	3.2 (3)	4/4 (160–320)
	10^3 PFU	None	2.9 (3)	4/4 (160–320)
	10^3 PFU	IFN	4.1 (3)**	2/4 (80–160)
H5N1	10^3 EID_{50}	None	4.2 (1)	4/4 (40–80)
	10^3 EID_{60}	IFN	3.4 (3)*	2/4 (20–40)

[a] IFN treatment was given on days −1, 0, 1, 3, 5, and 7 p.i.
[b] Expressed as mean log PFU/EID_{50} (day of observation). *, virus was detected in only 1/4 animals; **, virus was detected in only 2/4 animals.

virus). Collectively, these findings show that at low virus challenge doses, IFN treatment is capable of blocking replication of 1918 and H5N1 viruses in the guinea pig model.

To determine the ability of IFN treatment to control virus replication following a more stringent virus challenge, four animals were treated with a similar regimen of prophylactic human IFN following inoculation with a greater dose (10^6 PFU) of 1918 virus. In this case, while virus was recovered in the nasal washes of all IFN-treated animals, viral titers were over 100-fold lower on day 1 p.i. in animals receiving IFN treatment than those observed in PBS-treated controls (Fig. 4C and D; Table 2). This finding demonstrates that IFN treatment, while not able to block influenza virus infection at a high virus input dose, can reduce virus replication in the upper airway early after virus exposure.

In addition to evaluation of virus titers, we examined the ability of IFN treatment to prevent virus-induced pulmonary lesions following H5N1 infection. Less severe pneumonia was observed in animals treated with IFN prior to infection. Moderately severe bronchopneumonia was observed in untreated, H5N1-infected animals (Fig. 5A), while the pathology observed in treated animals was mild (Fig. 5B). It is important that IFN treatment alone did not result in signs of clinical illness or gross pathology in the lung tissue sections examined (data not shown).

Reduction of H5N1 virus titer following a single treatment of guinea pigs with recombinant human IFN. Given the ability of multiple doses of IFN to prevent highly pathogenic influenza virus infection in guinea pigs, we next evaluated the ability of a single dose of prophylactically administered IFN to control H5N1 virus infection. In this experiment, recombinant human IFN or PBS was instilled by intranasal administration approximately 12 h prior to inoculation with 10^6 (Fig. 6A) or 10^3 (Fig. 6B) EID_{50} of H5N1 virus. Following inoculation of guinea pigs with 10^6 EID_{50} of H5N1 virus, IFN-treated animals showed over 300-fold lower nasal wash viral titers on day 1 p.i., with titers remaining lower than those in untreated animals through day 3 p.i. (Fig. 6A). At subsequent time points, on days 5 and 7 p.i., virus was recovered at elevated titers from nasal washes of IFN-treated animals, while titers in PBS-treated control animals declined. A similar pattern of virus replication was observed with the reduced 10^3 EID_{50} challenge dose of H5N1 virus (Fig. 6B), despite virus titers recovered from nasal washes that were generally reduced compared with those for animals receiving a higher virus challenge dose. IFN-treated animals showed no detectable virus in nasal washes on day 1 p.i., at a time when control animals showed an average titer of 10^4 PFU/ml. However, virus levels rebounded in treated animals after day 1 p.i., while virus titers had declined in control animals at this time (Fig. 6B). Collectively, these studies suggest that a single dose of IFN is capable of reducing the replication of a clade 1 avian H5N1 isolate for approximately 2 to 3 days.

DISCUSSION

Influenza virus pathogenesis has commonly been studied in the laboratory mouse and the domestic ferret. In these animal models, influenza virus isolates are frequently grouped into two classes based on the severity of clinical signs that they induce and the tissue tropism of the virus (6, 13, 15, 31, 42, 44, 86). Viruses of low pathogenicity in mice and ferrets generally cause mild illness, with virus replication limited to the respiratory tract; morbidity is characterized by weight loss of <10%, and virus-induced clinical signs, such as ruffled fur, elevated temperatures, and lethargy following infection, are infrequently observed. Viruses of high pathogenicity typically induce high morbidity (weight loss of >10%) and mortality and are frequently associated with virus spread from respiratory tissues to the brain, spleen, or blood. It should be noted that not all highly virulent influenza viruses spread systemically; the 1918 virus is highly lethal in inoculated ferrets and mice, but virus replication could not be demonstrated in extrarespiratory tissues (73; unpublished observations).

The guinea pig has recently emerged as an alternate model with which to study the transmission of influenza viruses (40). While ferrets remain the ideal model to study many aspects of influenza virus infection, the higher cost and large amount of space required for housing this species severely limit the number of animals that can be included in individual studies (41). Given their relatively low cost and reduced size, guinea pigs represent a suitable alternative host for studies requiring large numbers of animals or multiple treatment groups (41). Thus far, studies with guinea pigs have largely been performed with nonvirulent human H1N1 or H3N2 strains. In the current study, we assessed the virulence of two avian influenza virus strains as well as two human influenza viruses of different pathogenicities. We found that infection of guinea pigs with the low-pathogenicity viruses Dk/Alb and Tx/91 resulted in only minimal weight loss, lethargy, and changes in temperature during the course of infection (Table 1). Surprisingly, both 1918 and H5N1 viruses, which exhibit high levels of virulence in mice, ferrets, and macaques (33, 44, 73, 75), did not induce significant morbidity or mortality among inoculated guinea pigs, despite multiday virus shedding in the upper airway. While minimal weight loss was observed following H5N1 virus infection, the 1918 virus induced no morbidity at any time during the course of infection. Such a dramatic reduction of virulence of both H5N1 and 1918 viruses observed in this species allows for further studies to identify the host genetic factors responsible for the resistance to lethal influenza virus infection. It is possible that the functional Mx proteins of guinea pigs may limit virus virulence, as the high-pathogenicity phenotype generally associated with these viruses was abro-

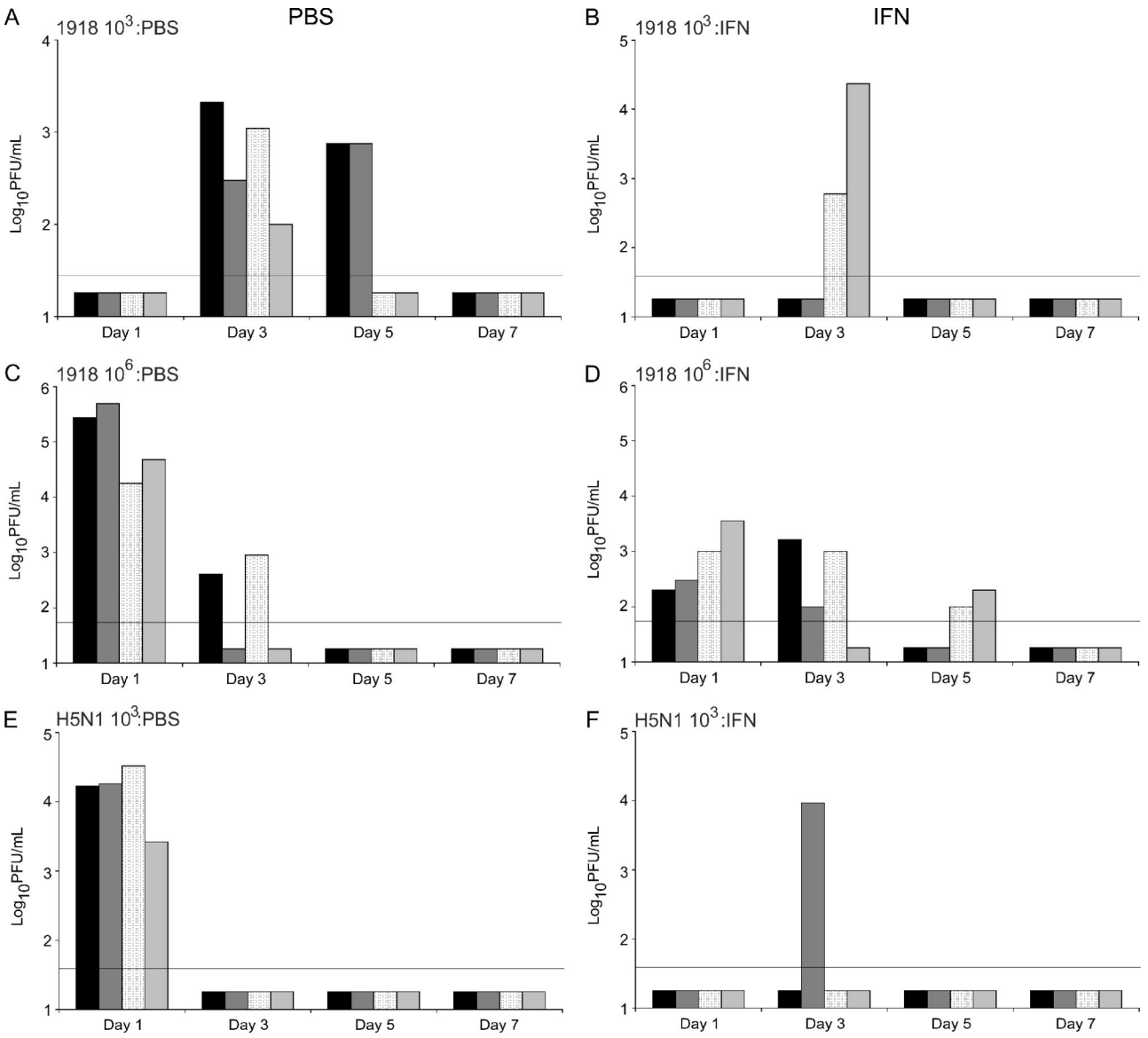

FIG. 4. Replication of highly pathogenic H5N1 and H1N1 influenza viruses in the upper airway in control and IFN-treated guinea pigs. Viral titers in nasal washes were determined following intranasal inoculation of four animals with the 1918 pandemic virus (A to D) or an H5N1 virus (E and F). Virus titers from untreated control animals (A, C, and E) were compared to those observed following multiple IFN treatments on days −1, 0, 1, 3, 5, and 7 p.i. (B, D, and F). Titers from individual animals are presented. (A) At a challenge dose of 10^3 PFU, 1918 virus could be detected in 4/4 untreated control animals but was detected in only 2/4 IFN-treated controls. (B) Following challenge with 10^6 PFU of 1918 virus, virus could be detected in control (C) and IFN-treated (D) animals, although the titers were lower in IFN-treated animals. Similar findings were obtained following challenge with 10^3 EID_{50} of H5N1 virus. Virus was detected in 4/4 control animals (E) but was detected in only 1/4 IFN-treated animals (F).

gated in mice possessing a functional Mx gene (59, 76). However, both viruses tested here show a high-pathogenicity phenotype in ferrets, which, like guinea pigs, possess a functional Mx gene (40; P. Staeheli, unpublished observations).

The lack of widespread severe lung lesions induced by highly pathogenic influenza virus isolates in guinea pigs may help to explain the lack of morbidity and mortality observed in this species. In mice as well as ferrets, both H5N1 and 1918 viruses have been found to induce moderate to severe necrotizing bronchitis as well as alveolitis throughout multiple lung lobes (44, 73, 75). Moreover, the distribution of histopathologic lesions mirrors the distribution of viral antigen, which can be observed in both the bronchial epithelium and throughout the lung parenchyma in alveolar cells. In general, influenza virus replication in the lung is proposed to lead to acute lung injury, ultimately resulting in fatal, severe acute respiratory distress syndrome (71). However, the lung lesions in guinea pigs infected with H5N1 or 1918 virus were milder than those reported for infected mice and ferrets and were mainly restricted to the parenchyma surrounding bronchioles in the hilar zone of the dorsal area of multiple lung lobes. Antigen was only rarely visualized in bronchiolar epithelium and was not found in

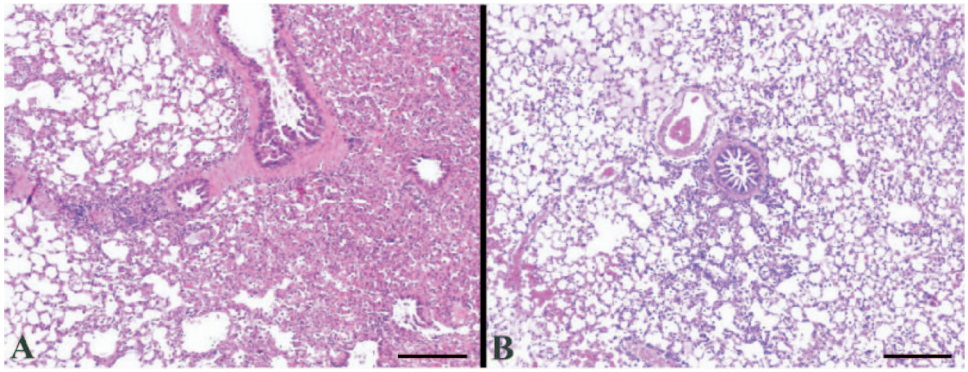

FIG. 5. Photomicrographs of hematoxylin- and eosin-stained tissue sections from PBS- and IFN-treated guinea pigs. (A) Moderately severe interstitial pneumonia adjacent to a large bronchiole and less severe pneumonia in the distal portion of the lung lobe area at 3 days p.i. in an animal challenged with H5N1 virus alone. Bar = 250 μm. (B) Mild focal interstitial pneumonia with alveolar edema at 3 days p.i. in an H5N1 virus-infected animal receiving IFN treatment. Bar = 250 μm.

alveolar cells. This lack of virus antigen, along with moderate lung titers and the focal lung pathology among H5N1 and 1918 virus-infected guinea pigs, suggests that sites of virus replication are more restricted in the guinea pig lung than in other animal models. This restriction may, in turn, prevent extensive lung injury, ultimately resulting in a decrease in morbidity and mortality following infection with highly pathogenic influenza viruses. A similar presence of mild pulmonary lesions and lack of clinical signs were seen in guinea pigs intranasally inoculated with another clade 1 (A/Vietnam/1203/04) and a clade 2.3 (A/Muscovy duck/Vietnam/209/06) H5N1 virus (34).

Successful infection of the mammalian respiratory tract by influenza viruses is most likely due to multiple factors that include host receptor expression. Many studies have focused on the identification of influenza virus target cells in the mammalian airway, using lectins that specifically bind the sialic acid moieties that serve as receptors for influenza virus. Collectively, this work has shown that the receptors for avian and human influenza viruses are asymmetrically distributed throughout the respiratory tract (26, 48, 63, 80). In general, avian and human influenza virus receptor distributions correlate with the abilities of tissues to support virus replication in vivo and ex vivo (49) and with the binding of labeled virus particles or proteins to tissue sections (64, 79), although it should be noted that replication of avian viruses has been observed in excised tissue lacking obvious receptors (49). Thus far, the influenza virus receptor distribution in the guinea pig respiratory tract has not been investigated, although studies investigating the sialic acid distribution have been carried out in other contexts (21, 29, 43, 51). The fact that avian (with an α2-3 sialic acid receptor binding preference) and human (with an α2-6 sialic acid receptor binding preference) viruses could be isolated at similar high titers from nasal washes suggests that both types of sialic acid receptors are present in the guinea pig respiratory tract. In ferrets and in human tissue, the α2-6-linked receptors that are bound by human influenza viruses are restricted primarily to the upper respiratory tract, whereas the α2-3-linked receptors preferentially bound by avian viruses are more abundantly present in both the lung parenchyma and the respiratory epithelium (36, 48, 63).

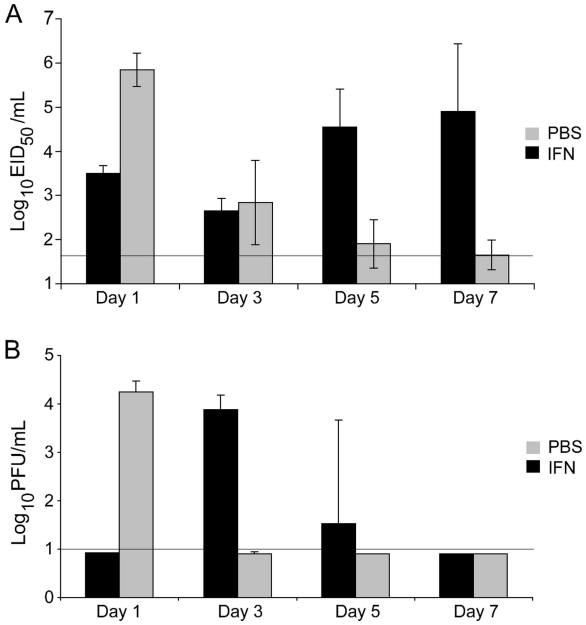

FIG. 6. Replication of H5N1 virus in the upper airway in guinea pigs following a single-dose IFN treatment. Virus titers in nasal washes were determined on days 1, 3, 5, and 7 p.i. following challenge with 10^6 (A) or 10^3 (B) EID_{50} of H5N1 virus. A single dose of exogenous IFN was administered intranasally 24 hours prior to intranasal inoculation of virus. Mean titers were plotted ($n = 5$), with error bars representative of standard deviations from the means.

IFN treatment is currently employed in the treatment of chronic hepatitis C virus infection and has also shown promise as a potential therapeutic for other viruses, such as hepatitis B virus and the severe acute respiratory syndrome coronavirus (32, 54, 83). Recently, Beilharz et al. showed that a low oral dose of IFN-α delivered daily as prophylactic therapy protected C57BL/6J mice against lethal challenge with a mouse-adapted H1N1 virus (5). In another 2007 study, a single prophylactic IFN treatment given intranasally was effective in reducing the virus titer following H5N1 challenge, but this effect was dependent on the Mx protein; a single IFN treatment significantly reduced the titer of A/VN/1203/04 (H5N1) virus in $Mx^{+/+}$ but not $Mx^{-/-}$ mouse lungs (76). In the guinea

pig model, we show here that IFN treatment effectively induces Mx protein expression and reduces virus shedding following infection with H5N1 or H1N1 highly pathogenic viruses. This was demonstrated using a high virus challenge dose of the virulent H5N1 isolate, where virus shedding was reduced over 100-fold as early as day 1 after infection. Using a reduced virus challenge dose, the effect of IFN was even more pronounced, with virus titer reductions of at least 10,000-fold observed. This titer reduction can be correlated with the expression pattern of the guinea pig Mx protein observed following a single dose of exogenous IFN. At day 3 p.i., an increase in virus titers coincident with a declining Mx level was observed in animals receiving a single-dose IFN treatment (Fig. 3). When the IFN treatment regimen was expanded to include multiple IFN doses, infection was fully blocked in 50% of challenged animals, as determined by a lack of viral isolation and absence of seroconversion to the inoculating virus. The studies presented here assessed the use of IFN as a prophylactic treatment to reduce the viral load postinfection and suggest that it would be possible to block influenza virus infection with exogenous IFN. It will also be important in future studies to determine the efficacy of therapeutic IFN treatment initiated following infection to prevent both morbidity and subsequent transmission of influenza viruses. However, in considering the application of these studies to human treatment during a pandemic, it is important that IFN treatment in humans is associated with a number of adverse side effects (8, 45, 52), making multiple treatments with high doses of IFN potentially untenable. The appearance of frequent adverse events could be corrected by reducing the total dose of IFN provided during the course of treatment. The studies presented here suggest that the guinea pig is a useful model for testing the effects of lower doses of IFN in an attempt to develop multiple-dose treatment regimens that could be adapted for use in humans. Furthermore, while the exclusive use of high-dose IFN as an antiviral for treatment of highly pathogenic influenza virus infection in humans may be impractical, our results demonstrate that single high doses of IFN may be useful in suppressing virus titers and replication at early time points postinfection, which could allow for bioaccumulation of more specific influenza virus antivirals, such as oseltamivir. This transient reduction in titer may be useful in preventing transmission to uninfected persons, helping to slow the spread of virus during a pandemic. Finally, it will become important to investigate the use of IFN treatment, both therapeutic and prophylactic, in combination with other influenza virus-specific antivirals as a means of further limiting virus replication. Such combination therapies have been used in other models, with promising results (18, 27, 85), and may circumvent the potentially harmful effects of multidose high-level IFN treatment.

One of the important mechanisms by which the IFN response pathway establishes an antiviral state is through the establishment of a positive feedback loop in infected cells; binding of IFN to a cell results in increased production and secretion of IFN that can in turn act on both the producer cell and neighboring cells (47, 60; reviewed in reference 46). It is interesting that in our studies, H5N1 viruses seemed to be able to overcome this feedback loop. At both high and low virus challenge doses, virus replication was reduced or delayed by single-dose IFN treatment but did eventually rebound to reach levels similar to those observed in PBS-treated controls (Fig. 5A and B). In multiple IFN treatment studies, IFN was unable to prevent infection but did reduce titer. Similar findings have been observed in other antiviral studies demonstrating that high challenge doses may overwhelm exogenous or host antiviral responses, achieving efficient replication despite inhibition by antiviral compounds or by the host response (7, 58, 82). The NS1 proteins of H5N1 viruses have been shown to be especially potent suppressors of the IFN response, and reassortant viruses containing NS1 genes from these viruses show increased pathogenicity in mice compared to that of their parental strains (19, 61). The ability to overcome single-dose IFN treatment demonstrated here supports previous work with the murine system demonstrating that the NS proteins from H5N1 viruses are highly effective in countering the IFN system (28, 37, 38). Further dissection of the IFN response in guinea pigs and a detailed analysis of the NS1-mediated antagonism of this system, using H5N1 NS1-bearing reassortant viruses, would be necessary to further implicate and dissect NS1 as a virulence factor in guinea pigs.

In conclusion, the findings presented here seek to determine whether the guinea pig provides a useful small animal model for the study of influenza virus pathogenesis and whether this model could be used for development of antiviral treatments that could be tested against viruses with pandemic potential. We established that influenza viruses with low and high virulence are capable of infecting guinea pigs and that these isolates induce a spectrum of pathological changes in lung tissue similar to those observed in other animal models. However, the absence of severe disease observed following infection with highly pathogenic viruses suggests that guinea pigs may be of limited utility in the study of influenza virus pathogenesis. Nonetheless, the ability of the guinea pig to respond to exogenous IFN treatment suggests that this model would be useful in the development of novel therapeutic strategies, especially those which would be difficult or expensive to test in other, larger animal models. An important role of antiviral drugs during an influenza pandemic will be to slow virus replication and subsequent spread while an appropriate vaccine is in production.

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